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The Renal and Cardio Protective Effects of Aliskiren and Pentoxifylline Alone and in Combination on Streptozotocin Induced Diabetic Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NAEM and TAEM designed, wrote the protocol and supervised the study, author KIED supervised the study and performed the histopathological examination and immunohistochemistry and author SKG performed the experiments, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background/Aims: The aim of the present study is to investigate the renoprotective and cardioprotective effect of aliskiren and pentoxifylline, alone and in combination, on streptozotocin (STZ) induced diabetic rats.
Methods: Fifty male Sprague Dawley rats were divided into 5 groups of 10 rats each, namely the control (Group I), diabetes (Group II), diabetic treated with aliskiren (Group III), diabetic treated with pentoxifylline (Group IV), and diabetic treated with aliskiren-pentoxifylline combination (Group V). Aliskiren (10 mg/kg/day) and pentoxifylline (55 mg/kg/ day) were given by oral-gavage once daily for 8 weeks. Renal function, oxidative stress parameters, immunohistochemical detection for transforming growth factor β, and kidney and heart histology were determined.

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Results: Serum urea and creatinine reduced significantly in the three treated groups, Heart reduced glutathione (GSH) increased significantly only in aliskiren-pentoxifylline treated group, while kidney GSH increased significantly in the three treated groups. Kidney malondialdehyde (MDA) reduced significantly in the three treated groups, while heart MDA reduced significantly only in aliskiren-pentoxifylline treated group. No significant changes were detected in heart nitric oxide in all treated groups, while kidney nitric oxide reduced significantly in the aliskiren alone and aliskiren-pentoxifylline treated groups. Our results show that heart tumor necrosis factor alpha (TNF-α) reduced significantly only in the aliskiren-pentoxifylline treated group. On the other hand, kidney TNF-α reduced significantly in the pentoxifylline alone and aliskiren-pentoxifylline treated groups. Immunohistochemical detection of transforming growth factor β (TGF-β) in kidney and heart sections shows positive TGF-β and a high intensity of staining in the diabetic group, while it shows only mild to moderate staining in the three treated groups. Histopathological examination of kidney and heart sections shows more improvement in the aliskiren-pentoxifylline combination therapy than aliskiren and pentoxifylline monotherapy.
Conclusion: Combining aliskiren with pentoxifylline provided a greater reduction in heart and kidney damage than either drug alone in STZ induced diabetic rats.

Keywords: Aliskiren; pentoxifylline; aliskiren-pentoxifylline combination renoprotection; cardioprotection.

1. INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease which is characterized by an absolute or partial deficiency in insulin secretion and/or insulin action, leading to hyperglycemia [1]. As a consequence of chronic hyperglycemia various complications occur which may be acute or chronic. Diabetic ketoacidosis (DKA) and hyperglycemic hyperosmolar state (HHS) are notable acute complications of diabetes. The chronic complications are the main causes of the morbidity and mortality associated with the disease as they affect different organ systems, these chronic complications can be nonvascular or vascular [2]. The vascular complications of DM are further subdivided into macrovascular complications, like coronary artery disease (CAD), peripheral arterial disease (PAD), cerebrovascular disease, and microvascular complications like retinopathy, neuropathy, and nephropathy. Nonvascular complications include gastroparesis, infections and skin changes [3-6].

Several studies suggested that the renin-angiotensin-aldosterone system (RAAS) has a role in the pathogenesis of diabetes complications [7-10]. The RAAS blockade with angiotensin converting enzyme (ACE) inhibitors, angiotensin II receptor type $1(AT_{1})$ receptor-blockers, and renin inhibitors, was found to induce beneficial effects on diabetic complications. These findings suggest an involvement of the RAAS in diabetic organ affection [11-13]. Increased oxidative stress and inflammation are also believed to have a role in development of diabetes and its complications [14-16].

Aliskiren is a direct renin inhibitor (DRI), it acts at the point of activation of the RAAS, inhibiting the conversion of angiotensinogen into angiotensin I by renin [17]. Aliskiren was found to have an antiproteinuric effect in patients with diabetes and also has cardioprotective, renoprotective, and anti-atherosclerotic effects in animal models independent of its blood pressure lowering activity [18-20].

Pentoxifylline (PTX) is a nonselective phosphodiesterase inhibitor and a methylxanthine derivative which is used to treat patients with peripheral vascular disease [21]. In addition to its hemorheologic activity, it has been experimentally shown to have an antioxidative [22], potent antiproliferative, anti-inflammatory [23], and anti-diabetic effects [23,24].

This study is intended to investigate the renal- and cardio-protective effects of aliskiren and pentoxifylline alone and in combination on STZ induced diabetic rats.

2. MATERIALS AND METHODS

2.1 Drugs and Reagents

Pentoxifylline was purchased from Sanofi-Aventis Egypt. Aliskiren (Tekturna) was purchased from Novartis, US, and Streptozotocin was purchased from MP Biomedicals, France. All other drugs and reagents were of analytical grade.

2.2 Animals

All procedures were carried out in accordance with institutional guidelines for animal research. Experimental protocols had the approval of ethical committee, Faculty of pharmacy Tanta University, Egypt.

50 Male Sprague Dawley rats weighing 170-200 g, obtained from the animal house of Vaccines and Sera Company (VACSERA) Helwan, Egypt, were used in this study. Rats were housed in a controlled environment with a 12 hour light/dark cycle and were fed standard chow diet and distilled water *ad libitum.*

2.3 Experimental Design

Diabetes was induced after an overnight fast by a single intraperitoneal injection (i.p.) of 65 mg/kg streptozotocin (STZ) freshly dissolved in 10 mM citrate buffer (pH 4.2). After 48 hours, fasting Blood glucose levels were checked by a test strip glucometer (Gluco Dr. Auto, Allmedicus, Korea) using one drop of tail blood obtained by tail incision. Rats with a blood glucose level over 300 mg/dL were considered diabetic. Rats were randomly assigned to one of the following five groups:

(1). Control group (Group I): Non diabetic rats received vehicle.
(2). Diabetic group (Group II) (n=10): Rats received vehicle.
(3). Aliskiren group (Group III) (n=10): diabetic rats received Aliskiren 10 mg/kg/day dosage by oral gavage.
(4). Pentoxifylline group (Group VI) (n=10): Diabetic rats received pentoxifylline 55 mg/kg/day dosage by oral gavage.
(5). Aliskiren + Pentoxifylline group (Group V) (n=10): Diabetic rats received Aliskiren 10 mg/kg/day plus pentoxifylline 55 mg/kg/day dosage by oral gavage.

After 8 weeks rats were anesthetized, blood samples were collected by heart puncture, and kidney and heart samples were taken.

2.3.1 Determination of serum creatinine

Serum creatinine was measured by Jaffe's reaction using commercially available clinical assay kit (Diamond, Egypt).

2.3.2 Determination of urea

Serum urea was measured colorimetrically according to modified Berthelot reaction using commercially available clinical assay kit (Greiner, Germany).

2.3.3 Determination of reduced glutathione

Reduced glutathione was determined in heart and kidney spectrophotometrically, according to the method of Richardson and Murphy [25].

2.3.4 Determination of lipid peroxidation

Lipid peroxidation was assayed in heart and kidney using thiobarbituric acid method [26].

2.3.5 Determination of nitric oxide

Nitric oxide (NO) in heart and kidney was determined spectrophotometrically using vanadium (III) reduction method [27].

2.3.6 Determination of tumor necrosis alpha (TNF-α)

TNF-α was determined in heart and kidney using commercially available ELISA kit (Assay pro, USA).

2.3.7 Histopathological examination

2.3.7.1 Tissue processing and staining

Immediately after anesthetizing the animals, kidneys were separated into two halves, and immediately fixed in Bouin's fixative (aqueous saturated picric acid solution, formalin, and glacial acetic acid 15:5:1). Hearts were removed and immediately fixed in neutral buffered formalin 10%. Sections from kidneys and hearts were routinely processed in ascending grade of alcohol, cleared in xylene, and then embedded in paraffin wax to produce paraffin blocks.

2.3.7.2 Hematoxylin and eosin staining and histopathological examination

The paraffin blocks were serially sectioned into 5μm thick sections, and stained with hematoxylin and eosin. Stained sections were reviewed to assess the histological diagnosis.

2.3.7.3 Immunohistochemical staining

The immunohistochemical stains that was used is polyconoclonal anti-rat TGF-β (ABD Serotec, UK).

2.4 Statistical Analysis

Comparisons among different treatment groups were assessed by ANOVA, and was performed

using the software package SPSS, 18th version (SPSS Inc., Chicago, IL, USA). Data is expressed as mean ± standard error (SE). *P = .05* was considered statistically significant.

3. RESULTS

3.1 Serum Urea and Creatinine

As shown in Table 1 Serum creatinine increased significantly in the diabetic group when compared to control animals (p=0.01). On the other hand, this parameter decreased significantly in the aliskiren treated group, pentoxifylline treated group (p=.05), and aliskiren-pentoxifylline combination treated group (p=0.01) when compared to the diabetic group.

Serum urea level was significantly higher in the diabetic group than the control group (p=0.01). serum urea decreased significantly in the Aliskiren treated group, pentoxifylline treated group and the aliskiren-pentoxifylline treated group compared to the diabetic group (p=.05).

3.2 Oxidative Stress Parameters

As shown in Fig. 1 levels of reduced glutathione (GSH) in the kidney (p=0.01), and heart (p=.05), decreased significantly in the diabetic group compared to the control group. Levels of kidney GSH increased significantly in the aliskiren treated group, pentoxifylline treated group, and the aliskiren-pentoxifylline treated group compared to the diabetic group (p=.01). Administration of the aliskiren-pentoxifylline combination also increased heart GSH significantly when compared to the diabetic group (p=.05), while heart GSH didn't change significantly in the aliskiren and pentoxifylline monotherapy.

Our results show that kidney and heart malondialdehyde (MDA) levels increased significantly in the diabetic group compared to the control group (p=0.01). There is a significant decrease in kidney MDA for the three treated groups compared to the diabetic group (p=0.01). Similarly, heart MDA decreased significantly in the aliskiren-pentoxifylline treated group compared to the diabetic group, but no differences were seen in the aliskiren treated group and in the pentoxifylline treated groups compared to the diabetic group.

Kidney nitric oxide (NO) decreased significantly in the diabetic group compared to the control group (p=.05), whereas a significant increase in kidney NO was detected in the aliskiren treated and the aliskiren-pentoxifylline treated groups compared to the diabetic group (p=.05), while the level of kidney NO didn't change significantly in the pentoxifylline treated group. Similarly, heart nitric oxide levels didn't changes significantly in all treatment groups.

3.3 TNF-α

As shown in Fig. 2, heart and kidney TNF-α levels increased significantly in the diabetic group compared to the control group (p=.05). There were no significant differences in the heart and kidney TNF-α levels in the aliskiren treated group compared to the diabetic group. Similarly, the level of heart TNF-α didn't change significantly in the pentoxifylline treated group compared to the diabetic group, while the level of kidney TNF-α decreased significantly (p=.05). The heart and kidney TNF-α decreased significantly in the aliskiren-pentoxifylline treated group compared to the diabetic group.

3.4 Immunohistochemistry

Fig. 4 shows immunostaining of kidney with transforming growth factor beta (TGF-β) specific antibody. The diabetic group shows highly positive staining for TGF-β, while the aliskiren treated and pentoxifylline treated groups show mild to moderate positive staining for TGF-β. A mild positive staining for TGF-β is observed in the Aliskiren-pentoxifylline treated group.

Table 1. The effect of aliskiren and pentoxifylline alone and in combination on the levels of serum urea and creatinine in STZ induced diabetic rats

	Group I	Group II	Group III	Group IV	Group V
Serum creatinine (mg/dL)	1.4±0.12	2.44±0.1**[a]	1.9±0.06*[b]	1.98±0.1*[b]	1.84±0.09**[b]
Serum urea (mg/dL)	38.67±1.9	83.5± 3**[a]	65.94±2*[b]	58.83±2*[b]	56.89±0.7*[b]

Group I: Control, Group II: Diabetic received vehicle, Group III: diabetic received Aliskiren, Group IV: diabetic received Pentoxifylline, Group V: diabetic received Aliskiren + Pentoxifylline. (*) p= .05, (**) p= .01, (a) compared to Group I, (b) compared to Group II

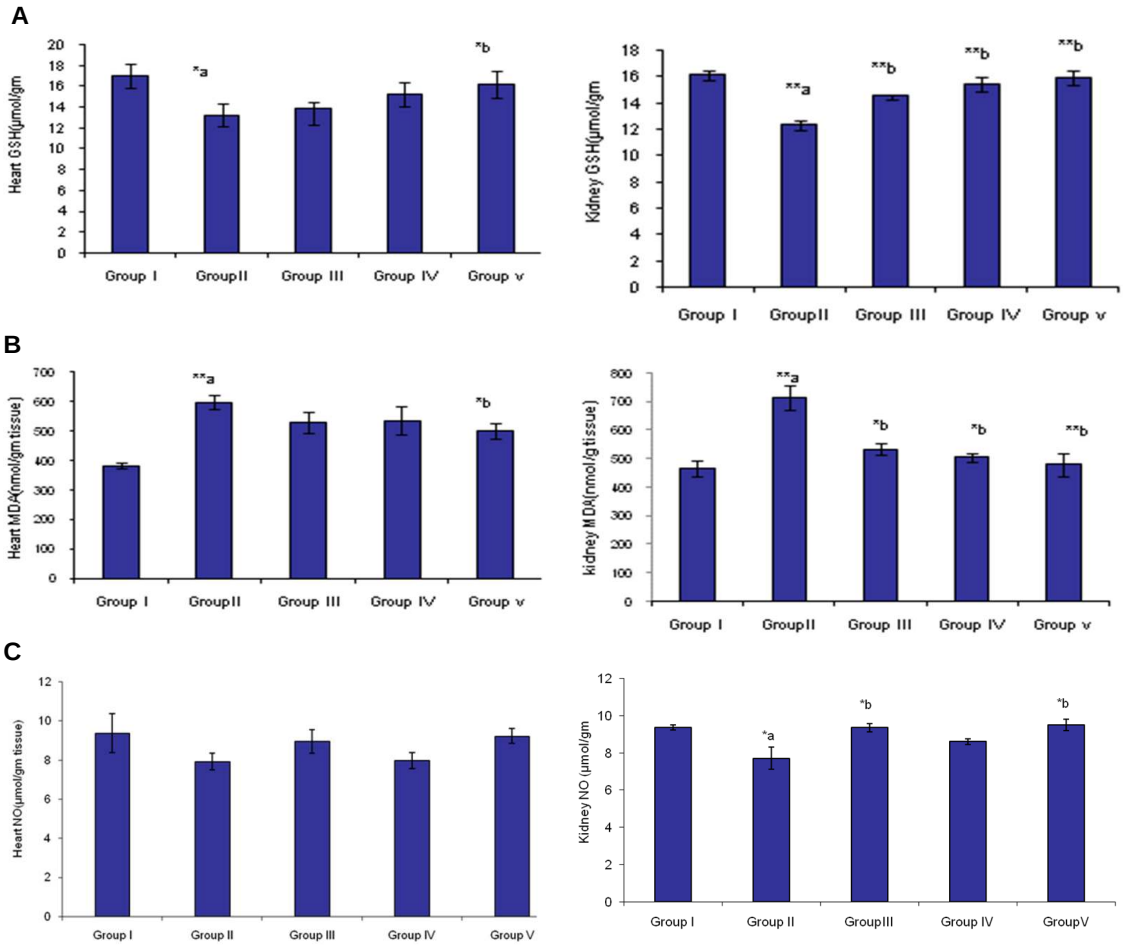

Fig. 1. The effect of aliskiren and pentoxifylline, alone and in combination, on the levels of renal and cardiac tissue GSH (A), MDA (B) and NO(C) in STZ induced diabetic rats; GSH: glutathione, MDA: malondialdehyde, NO: Nitric oxide

Group I: Control, Group II: Diabetic received vehicle, Group III: Diabetic received Aliskiren, Group IV: Diabetic received Pentoxifylline, Group V: Diabetic received Aliskiren + Pentoxifylline. (*) p=.05, (**) p=.01, (a) compared to Group I, (b) compared to Group II

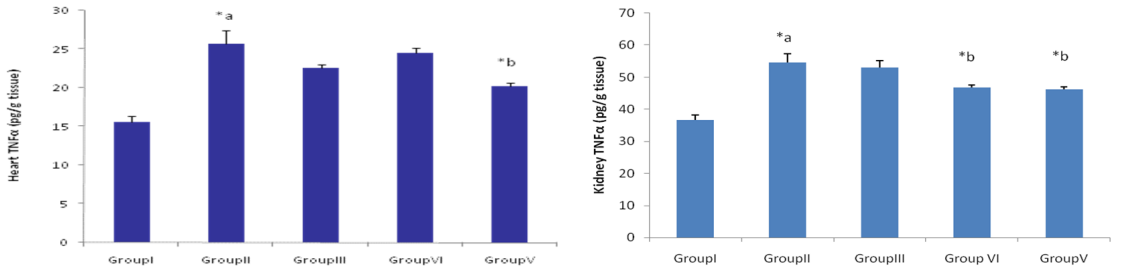

Fig. 2. The effect of aliskiren and pentoxifylline, alone and in combination, on the levels of renal and cardiac tissue TNF-α

Group I: Control, Group II: Diabetic received vehicle, Group III: diabetic received Aliskiren, Group IV: diabetic received Pentoxifylline, Group V: diabetic received Aliskiren + Pentoxifylline. (*) p=.05, (**) p=.01, (a) compared to Group I, (b) compared to Group II

Fig. 5 shows immunostaining of heart with TGF-β specific antibody. The diabetic group shows highly positive staining for TGF-β. On the other hand, the aliskiren, pentoxifylline, and the

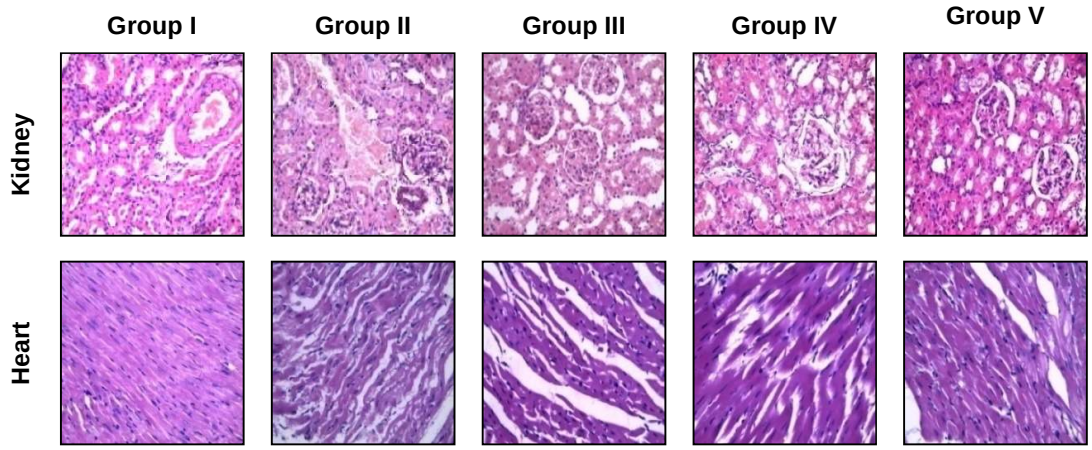

Fig. 3. Photographs of histological sections of the kidney and heart using hematoxylin and eosin (H&A) stain

Group I: Control, Group II: Diabetic received vehicle, Group III: diabetic received Aliskiren, Group IV: diabetic received Pentoxifylline, Group V: diabetic received Aliskiren + Pentoxifylline. All are ×200

aliskiren-pentoxifylline combination treated groups show mild to moderate positive staining for TGF-β.

3.5 Histopathological Findings

Fig. 3 shows histological sections of the kidney and heart using hematoxylin and eosin (H&A) stain. Histology of kidney in normal animals shows normal structure. Thickening in the basement membrane, the edematous proximal convoluted tubule and interstitial hemorrhage could be well observed in kidney section from the diabetic group. All these changes improved in the aliskiren and the pentoxifylline treated groups, while features of healing like a normal basement membrane, and the absence of any edema in the proximal convoluted tubule could be easily appreciated in the aliskiren-pentoxifylline treated group.

The hearts of the diabetic group show degeneration of some cardiomyocytes, with loss of myofibrils and disarray of muscle fibers. The aliskiren treated group and pentoxifylline treated groups were found to possess fewer severe histological changes in the cardiac tissues compared to the diabetic group, while the aliskiren-pentoxifylline treated group shows marked improvement of the degenerative changes of the myocardium, as the nuclei appeared nearly equal in size with uniform shape, and the cardiac muscle fibers had a regular array and intact myofibrils.

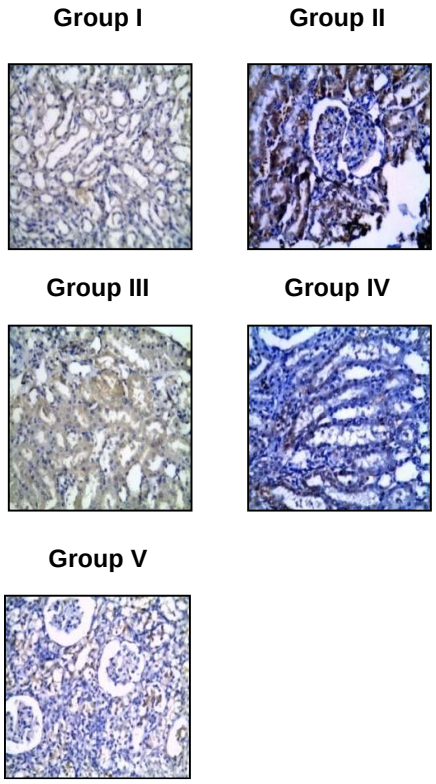

Fig. 4. Immunostaining of kidney with TGF-β specific antibody

Group I: Control, Group II: Diabetic received vehicle, Group III: diabetic received Aliskiren, Group IV: diabetic received Pentoxifylline; Group V: iabetic received Aliskiren + Pentoxifylline
Groups I, III, IV, and V are ×200, Group II is ×400

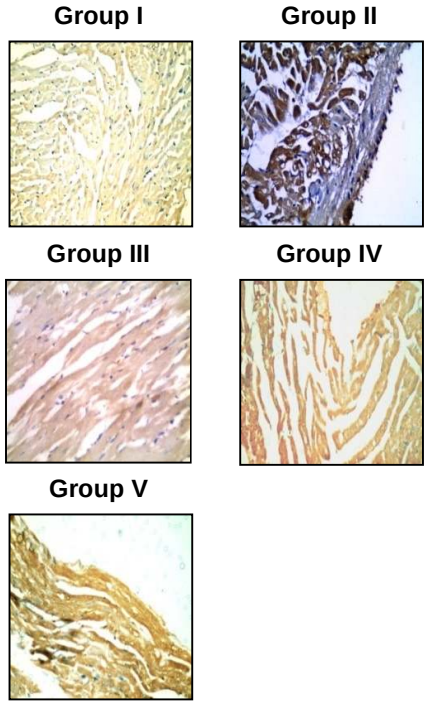

Fig. 5. Immunostaining of heart with TGF-β specific antibody

Group I: Control, Group II: Diabetic received vehicle; Group III: diabetic received Aliskiren, Group IV: diabetic received Pentoxifylline, Group V: diabetic received Aliskiren + Pentoxifylline.
Group I is ×100, Group II is ×400 Groups III,IV,V are ×200

4. DISCUSSION

This is the first study to assess the cardioprotective effect of aliskiren-pentoxiphylline combination in STZ induced diabetic rats. We also investigate the renoprotective effect of this combination in STZ induced diabetic rats. Several studies demonstrate that the RAAS plays a critical role in the development of diabetic complications [13-16], where plasma levels of renin and its precursor prorenin were found to increase in diabetes. The increased level of prorenin was found to be related to several pathological conditions, such as heart failure, diabetic nephropathy, and diabetic retinopathy [28,29].

In the current study, we found that aliskiren (10 mg/kg/day, administered for 8 weeks) significantly reduced serum creatinine and urea compared to diabetic rats. Several studies investigated the renoprotective effect of aliskiren in the animal models of diabetic nephropathy. Renin inhibition with aliskiren was found to prevent albuminuria, and suppress renal gene expression of the prorenin receptors in (mRen-2)27 transgenic rats [30]). In addition, Ghandhi et al. [31], reported that aliskiren reduced serum creatinine and serum cystatin significantly in STZ induced diabetic rats.

Our results also show that pentoxifylline (55mg/kg/day, administered for 8 weeks), significantly decreased serum creatinine and urea levels compared to diabetic rats. Pentoxifylline was reported to decrease proteinuria in patients with diabetic nephropathy [32]. It was also found to inhibit the renal inflammatory reaction, and prevent proteinuria significantly in the STZ diabetic rats [33].

The results reveal also that the aliskiren-pentoxifylline combination decreased the levels of serum urea and creatinine significantly, our results are in agreement with the results obtained by A.T. Hamed et al. [34], who investigated the renoprotective effect of aliskiren alone and in combination with pentoxifylline, in type 2-diabetic hypertensive patients with diabetic nephropathy, and have shown that an aliskiren-pentoxifylline combination (150, and 400 mg/day, respectively) significantly reduced urinary albumin excretion (UAE) rate after 6 and 9 months of treatment, and also reduced serum creatinine level throughout the study period.

Results obtained from both experimental and clinical studies suggest that oxidative stress plays a major role in the pathogenesis of diabetes mellitus. Activation of the RAAS in cardiomyocytes and endothelial cells in the hearts of diabetic patients was found to be correlated with increased oxidative stress, apoptosis, and necrosis [35]. Oxidative stress was also found to be involved in the pathophysiology of diabetic nephropathy. Local activation of the RAAS in diabetic tissue was found to contribute in ROS production [36-38].

Our results show that an aliskiren-pentoxifylline combination effectively reduced oxidative stress in both cardiac and renal tissues. It also significantly increased the levels of kidney and heart GSH, and kidney nitric oxide, while there was no significant change in the heart nitric oxide level. This combination was also able to reduce MDA levels significantly in both kidney and heart. Aliskiren alone and pentoxifylline alone were less effective in reducing oxidative stress in heart and kidney of STZ induced diabetic rats, both were able to increase the levels of kidney GSH and

reduce the levels of kidney MDA significantly. Furthermore, aliskiren was able to increase the level of kidney NO significantly. Neither aliskiren nor pentoxifylline alone significantly affected the levels of heart GSH, MDA and nitric oxide. These results are consistent with the findings of previous studies, where Aliskiren was reported to increase level of kidney nitric oxide in STZ induced diabetic rats. This increase in NO level was attributed to reduction of aldosterone level by aliskiren [39]. Furthermore, aliskiren was found to reduce cardiac superoxide and restore vascular eNOS production in db/db mice at low and high doses (3, 6, 12, 25 mg/kg/day) [40].

Pentoxifylline has been shown to induce an antioxidant effect in the kidneys of diabetic rats, to significantly reduce the levels of MDA, and to increase super oxide dismutase (SOD) levels in STZ induced diabetic rats [41]. Additionally, pentoxifylline was found to have an antioxidant effect and to exert renoprotective effects by decreasing kidney MDA levels and restoring intracellular glutathione in non-diabetic patients with chronic kidney disease [42].

It is well known that inflammatory cytokines such as IL-1, IL-6, and TNF- α are involved in the development of diabetic nephropathy [43]. TNF-α is also an important molecule that causes inflammation and cell injury in the heart, and consequently induce cardiac fibrosis [44]. Suppression of TNF-α has been shown to improve diabetic cardiomyopathy, reduce cardiac fibrosis, and enhance cardiac function in STZ induced diabetic rats [45].

The current study demonstrates that an aliskiren-pentoxifylline combination was able to significantly reduce TNF-α level in cardiac and renal tissues. Aliskiren alone didn't significantly affect the level of TNF-α in cardiac and renal tissue, while pentoxifylline alone significantly reduced TNF-α in renal tissue, but had no significant effect on the cardiac TNF-α levels. Our results are consistent with results of other studies. Pentoxifylline has been shown to inhibit the accumulation of TNF-α mRNA and the transcription of the TNF-α gene expression in kidneys of diabetic rats [46]. In addition, pentoxifylline was found to reduce urinary TNF- α levels when administered with RAAS blockers in patients with diabetic kidney diseases [24]. Wang et al. [47] demonstrated that aliskiren (25/kg/day) significantly reduced proinflammatory cytokines TNF-α, monocyte chemoattractant protein-1(MCP-1) and interleukin -1-β (IL1-β) in the kidneys of the diabetic mice.

Transforming growth factor-β (TGF-β) is an essential cytokine in the progression of fibrosis and/or sclerosis in the kidney and other organs [48]. Recent studies demonstrate that renin and its precursor, prorenin, may contribute to tissue damage via binding to and activating the renin/prorenin receptors, which leads to increased expression of profibrotic pathways and molecules, such as (TGF-β_1), plasminogen activator inhibitor (PAI-1), fibronectin, and collagen [49]. Diabetic cardiomyopathy is also characterized by interstitial and perivascular fibrosis with increased gene expression of TGF-β [50].

Our results show that the aliskiren-pentoxifyllyne combination reduces the production of TGF-β in cardiac and renal tissues. Results obtained from the aliskiren and pentoxifylline treated groups also show improvement, but to a lesser extent than the combination treated group. These results are consistent with results from other studies, where treatment with aliskiren was found to significantly reduce the gene expression of TGF-β, CTGF and PAI-1, therefore preventing kidney injury in diabetic mice [47]. Furthermore, aliskiren has been shown to suppress the synthesis of TGF-β through decreasing the vascular endothelial growth factor (VEGF) [31]. Pentoxifylline has also been shown to reduce the over-expression of TGF-β_1 and connective tissue growth factor (CTGF) in the remnant kidney [51]. In the current study histopathological examination of kidney and heart sections have shown improvement after the administration of aliskiren-pentoxifylline combination therapy for 8 weeks, which could be attributed to the reduction of oxidative stress and the inhibition of TNF-α and TGF-β formation by this combination. The effect of aliskiren on prorenin receptors could also partially contribute to the cardio and renoprotective effects of aliskiren- pentoxifylline combination , aliskiren was found to suppress prorenin receptors expression in diabetic TG(mRen-2)27 rats [30], elevated plasma prorenin concentration has been reported to be associated with microalbuminuria [52] and diabetic nephropathy [53]. In addition, aliskiren was found to significantly inhibit the diabetes-increased expression of prorenin receptors in the hearts of STZ induced diabetic mice [54].

5. CONCLUSION

In conclusion, our results demonstrated that the aliskiren-pentoxifylline combination is effective to alleviate renal and cardiac damage in STZ

induced diabetic rats. Aliskiren and pentoxifylline alone had less cardio- and renal-protective effect than the combination. Further animal and clinical studies are needed to confirm these findings.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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